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Significance of Basal Lamina for Regeneration of Injured Lung

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Summary. To study the relationship between basal lamina and regenerating cells, lungs of dogs were injured with intravenous injection of oleic acid. The lesion produced is characterized by desintigration of cells and by preservation of basal lamina. Within three days, regeneration with epithelial and endothelial cells, and probably with septal cells begins from the adjacent viable portions of the lung. Guided by the epithelial and endothelial basal lamina, these cells proliferate along the basal lamina surfaces, which have been denuded of cells, and in three weeks almost completely re-establish the structure and function of the lung.

The new epithelial and endothelial cells grow directly upon the original basal lamina and do not deposit a new layer.

Observations are presented which indicate that specific recognition systems may exist between denuded basal lamina and regenerating cells.

Zusammenfassung. Zur Überprüfung der Beziehungen zwischen Basalmembranen und regenerierenden Parenchymzellen werden die Lungen von Hunden durch intravenöse Injektion von Ölsäure geschädigt. Diese Schädigung ist gekennzeichnet durch eine Desintegration der Alveolarzellen, während die Basalmembranen erhalten bleiben. Die Regeneration der Epithelien, Endothelien und wahrscheinlich auch der septalen Zellen geht von den noch erhaltenen Parenchymbezirken aus. Die Basalmembranen dienen den regenerierenden Zellen als *Leitstrukturen*. Sie sind zugleich verantwortlich für die Offenhaltung der Alveolarräume. Nach 3 Wochen ist die Regeneration beendet und der status quo ante wieder erreicht. Die engen Beziehungen zwischen Basalmembranen und Regenerationsvorgängen machen es wahrscheinlich, daß zwischen regenerierenden Zellen und Basalmembranen spezifische Erkennungssysteme bestehen.

The significance of basal lamina (BL) for orderly regeneration of injured tissues has been revealed by Oliver (1953), O'Daly and Imaeda (1967), Cuppage *et al.* (1967, 1969), Giacometti and Parakkal (1969), Vracko (1970) and Krawczyk (1971). If BL is undamaged, it retains as a microskeleton the structural framework of the organ and repopulation with new generations of cells proceeds along the denuded BL surface. The result is almost complete restoration of structure and function. If continuity of BL has been damaged or destroyed (Clark, 1946; Oliver, 1953; Volkmann, 1893) regeneration is incomplete and scar ensues.

During repair of renal tubules (Cuppage *et al.*, 1967, 1969), cutaneous nerves (O'Daly and Imaeda, 1967) and skeletal muscle (Price *et al.*, 1964; Vracko, 1970) the new cells produce a new layer of BL in apposition to the old one. Such structures then have a two layered Bl. Multiple layers of BL may accumulate from successive generations of cells causing a marked widening of the overall BL investment (Vracko, 1970, 1970).

The preservation of BL in injured tissues and its ability to guide the proliferation of regenerating parenchymal cells constitute important biologic principles

which appear to be responsible for the final outcome of the healing process. They determine, according to this concept, whether the injury will heal by regeneration, re-establishing structure and function, or will terminate in scar. These qualities of BL also affect the development of BL thickening, as it occurs for example in patients with diabetes mellitus (Vracko, 1970, 1970).

To obtain additional information about the relationship between BL and regenerating cells the *healing of injured lung* was investigated. This tissue was chosen because it has relatively simple structure and because it possesses two distinct types of BL: one which supports epithelial cells and another belonging to capillary endothelium. The purpose of this communication is to: 1. show that after death of parenchymal cells BL of pulmonary septi provides a scaffolding for replacement of cells, 2. demonstrate that cell replacement in the lung does not result in thickening of BL and 3. discuss the significance and specificity of BL for regenerating cells. Chemical injury with intravenously administered oleic acid was used to injure and kill cells of alveolar septi. The injury and its healing were studied grossly and with light and electron microscopy.

Materials and Methods

Polyethylene No 280 catheters were placed into the femoral artery and into the external jugular vein of seven adult mongrel dogs. The following day 0.15 ccm/kg body weight of oleic acid (J. T. Baker Chem. Co.) were injected rapidly into the venous circulation of six dogs and saline was injected into one control animal. They were sacrificed at five hours (one dog), seventy-two hours (two dogs), one week (one dog), two weeks (one dog), and three weeks (one dog) after injection. The control animal was sacrificed seventy two hours after injection of saline. Samples of myocardium, spleen, liver, kidneys and from each lobe of the lung were fixed by immersion into neutral 10% formalin and embedded in paraffin. The sections were stained with H and E, PAS, and Gomori's trichrome stain. Frozen sections of lung embedded in 1% gelatine were stained with Oil-red-0.

For electron microscopy tissue samples were obtained from three randomly selected areas of left and right lungs, minced into 1 mm^3 pieces, fixed for one hour in prechilled 1% osmium tetroxide in 0.1 M s-collidine buffer at pH 7.48, postfixed in 10% neutral formalin for one hour, dehydrated in ethyl alcohol and embedded in Epon 812 via propylene oxide. For thin sectioning, five blocks showing abnormal lung tissues on thick sections were selected from each case and multiple thin sections were prepared, stained with 3.5% aqueous uranyl acetate and with Millionig's lead tartrate. Electron micrographs were obtained with RCA-EMU-3 G electron microscope.

Just prior to the injection of oleic acid as well as at 1, 3, 5, 24, 30 hours and 2, 3, 4, 5, and 6 days after the injection the following functional parameters were measured: respiration rate, minute ventilatory volume using the Wright's ventilometer with face mask, pH, pO_2 and pCO_2 of the arterial blood, pulse rate, and intra-arterial blood pressure.

Results

Immediately after injection of oleic acid all animals, except the control, developed respiratory distress. The respiration rate increased 2–3 times and the minute respiratory volume doubled within the first 3 hours. Arterial pO_2 decreased to 40–60 mm/Hg and the pH dropped from 7.41 to 7.37. The pulse rate increased on the average 15 beats per minute. These parameters remained abnormal for the first two days. They began to improve thereafter and returned to normal levels by the fourth day. Intra-arterial blood pressure and pCO_2 remained unchanged throughout the experiments. The control animal which received saline showed no changes in these parameters nor did it reveal any pulmonary pathology.

Gross Pathology. The lungs of animals which were sacrificed during the first three days weighed 2.5 times normal. They were firm, rubbery and had markedly decreased crepitation. At one week and later their weight was normal (0.9% of total body weight) and they contained only scattered areas of firmess. At two and three weeks the gross appearance was normal.

Light Microscopy. At five hours the changes were focal, characterized by intra-alveolar hemorrhages, edema, and infiltration with polymorphonuclear leukocytes. The distribution of lesions was throughout the lung and was not limited to subpleural parenchyma. Minute lipid droplets were scattered in many cells.

At seventy-two hours the areas of necrosis became defined more clearly. They were surrounded by pleomorphic cells and clusters of polymorphonuclear leukocytes. Pleural reaction was absent. Considerably more stainable lipid was present in various cells than was noted in the earlier specimen.

At one week more air-filled spaces were apparent. They contrasted sharply with areas of obliterated lung in which increase in fibrous tissues was apparent mixed with large pleomorphic cells with prominent nuclei and nucleoli. More stainable lipid was present than was seen on the seventy-two hour specimen. Disruptions of alveolar septi became apparent suggesting emphysema.

At two weeks the airless areas had diminished further. They contained more collagen and pleomorphic cells. PAS positive, brown pigment was noted now in alveolar macrophages. Inflammatory cells were absent and lipid occurred only in clusters of cells within areas of consolidation. Emphysema became prominent.

At three weeks only microscopic foci of fibrosis remained. The majority of parenchyma was normal except for common interruptions of interalveolar septi giving the picture of emphysema.

No pathologic changes were seen in the myocardium, spleen, liver or kidneys of any of the animals.

Electron Microscopy. The focal nature of the lesion was apparent also in tissues prepared for electron microscopy. While an occasional tissue block contained normal lung, most of the samples obtained during the first three days had changes ranging from mild interstitial edema and focal hemorrhage to areas of total necrosis. Among random samples obtained at one week and later, normal appearing lung became more common and comprised most of the tissue in the last sample.

Samples taken at *five hours* revealed flocculent intra-alveolar material mixed with occasional strands of fibrin, scattered erythrocytes and a rare white cell. Some alveolar septa exhibited widening of their interstitial spaces, apparently due to accumulation of fluid; others revealed partial detachment of epithelial cells from epithelial BL, forming bullae filled with flocculent material. The capillaries in these septa were intact and distended with red blood cells. The most severe damage was characterized by total loss of all cells (Fig. 1) except red blood cells. Epithelial, endothelial and interstitial cell remnants in these areas were present as granular debris. No lipid was seen within capillaries but an occasional lipid droplet occurred in the alveolar space among cell debris. The epithelial and endothelial BL remained unaltered and could readily be identified and differentiated by their relation to interstitial collagen and elastic fibers.

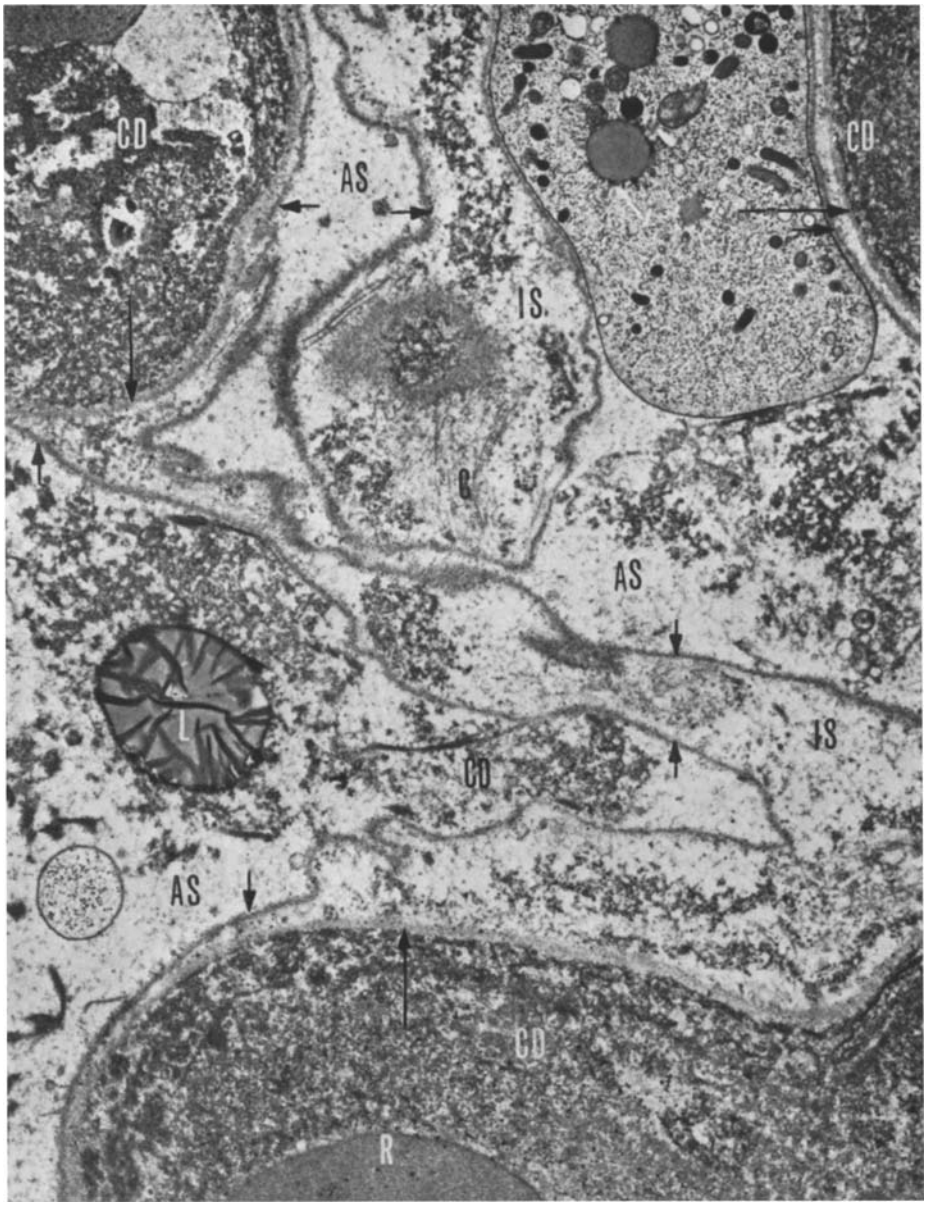

Fig. 1. Five hours after injury. Cell debris (*CD*) surrounds the undamaged epithelial (short arrows) and endothelial (long arrows) basal lamina. A glycogen rich cell, lipid droplet (*L*) and cell debris (*CD*) are present in alveolar spaces (*AS*). Collagen (*C*) identifies the interstitium. Irregular thickening of basal lamina is due to oblique sectioning. Lead tartrate and uranyl acetate. × 11000

Three days after injury considerable variation among samples was again noted. Some areas were relatively normal. In others, septi were surrounded by fibrin, pneumocytes and extravasated red blood cells. Foci with necrotic cells and

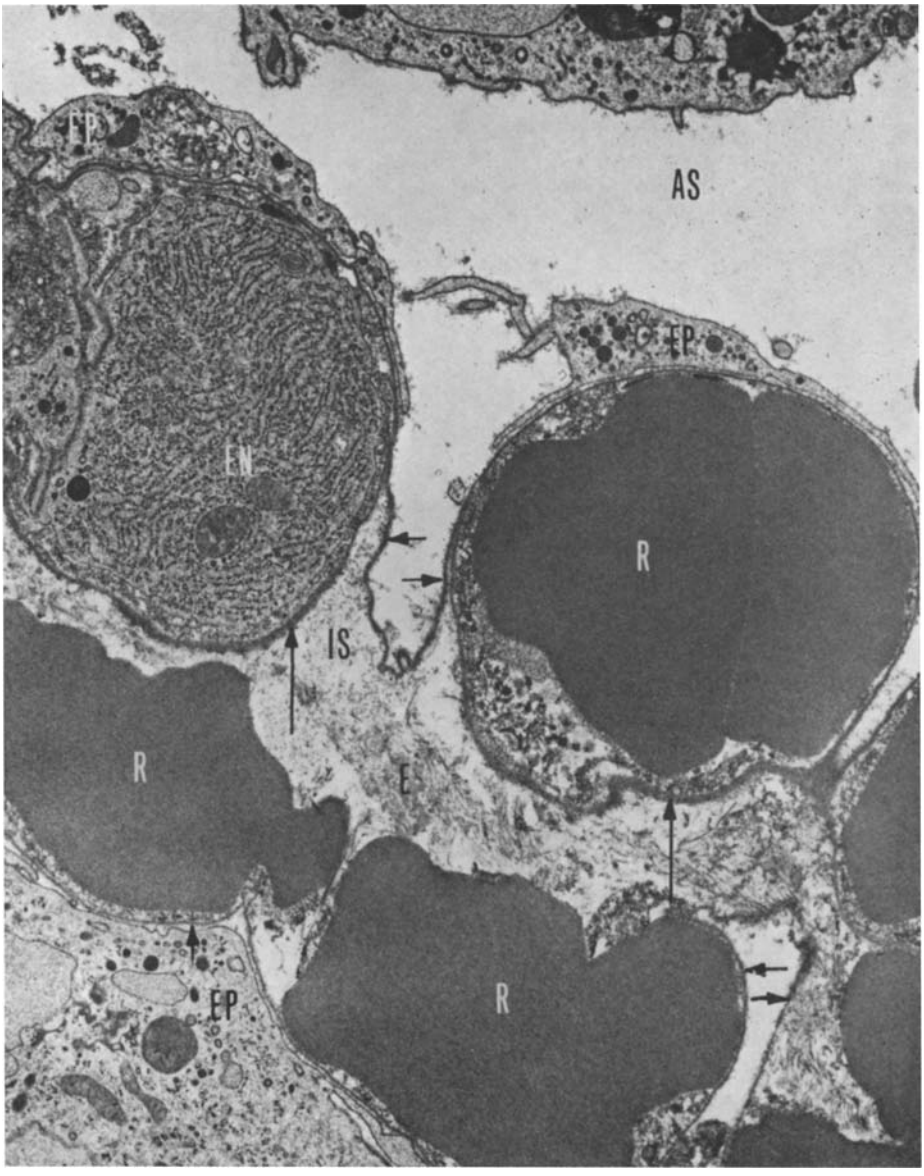

Fig. 2. Three days after injury. The basal lamina skeleton is being repopulated with epithelial cells (*EP*) along epithelial basal lamina (short arrows) and by endothelium (*EN*) inside capillary basal lamina (long arrows). Red blood cells (*R*) and cell debris fill out one capillary. Interstitium (*IS*) contains red blood cells (*R*), collagen, elastic fibers (*E*) and cell debris. Lead tartrate and uranyl acetate. × 9000

denuded, intact BL could be readily located. In these, new cells began to appear. Cells within the confines of capillary BL were conspicuous by prominent amounts of rough endoplasmic reticulum (Fig. 2, 3). Their cell walls were either in apposition with the luminal surface of capillary BL or with endothelial cell debris. They

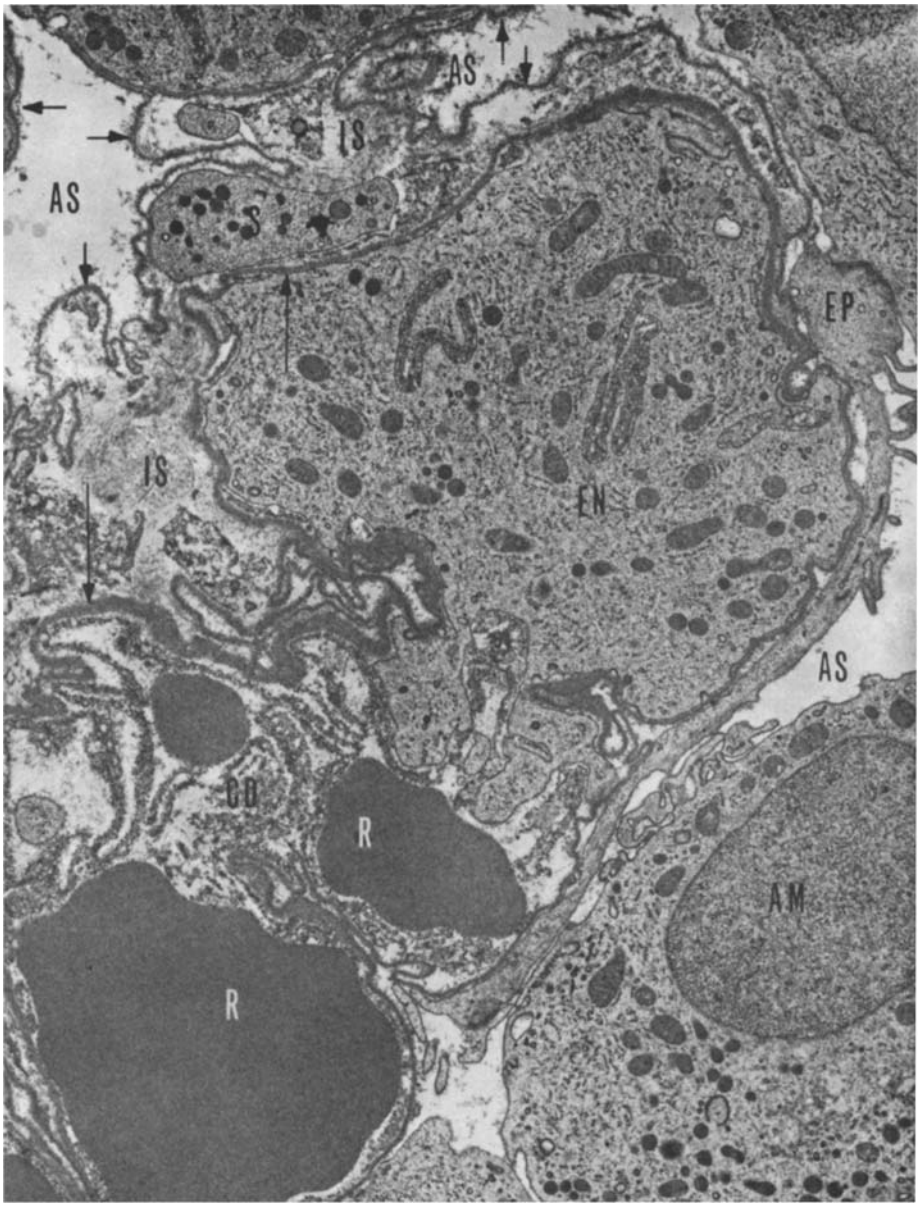

Fig. 3. Three days after injury. The partly collapsed but intact basal lamina skeleton is guiding regenerating endothelial (*EN*) and epithelial (*EP*) cells. A septal cell (*S*) containing electron opaque granules is present in the interstitial space (*IS*) and macrophages (*AM*) are seen in alveolar space (*AS*). Lead tartrate and uranyl acetate. × 10000

were not seen extending through BL. A different cell type was seen on the alveolar surface of epithelial BL (Fig. 2, 3): from a thickened portion of the cell which contained sparce cytoplasmic organelles, relatively thin cell processes extended

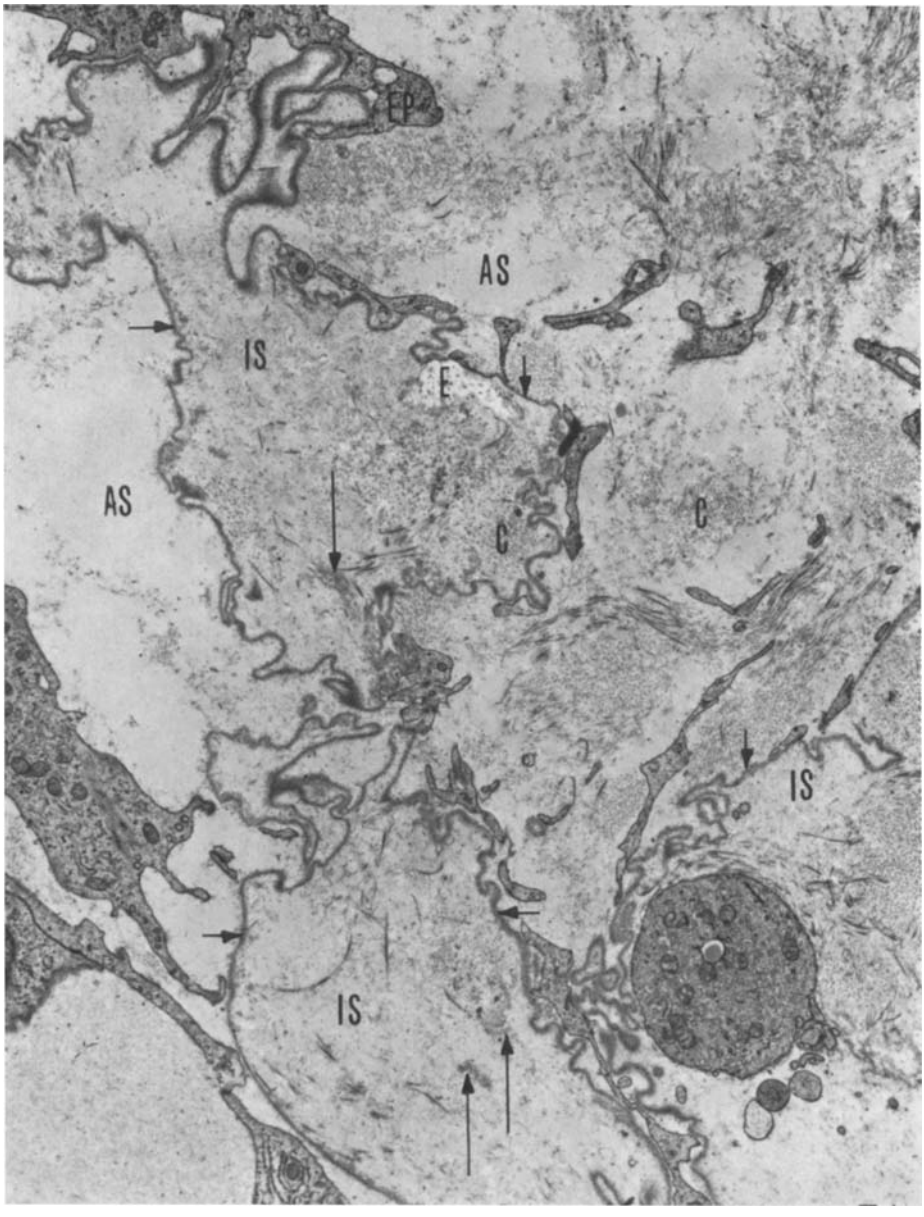

Fig. 4. A minute focus of fibrosis three weeks after injury. Elastic fibers (*E*) define the interstitium (*IS*). The long arrows point to probable remnants of capillary basal lamina. The alveolar spaces (*AS*) contain collagen (*C*) and cell processes, some of which are aligning along the epithelial basal lamina (short arrows). Lead tartrate and uranyl acetate. × 6500

over the alveolar surfaces of epithelial BL. These were almost totally devoid of cytoplasmic organelles. They bridged across BL folds and frequently extended shorter cytoplasmic processes into the depth of these BL pleats. In the early

stages of regeneration cell debris was commonly seen between the cell processes and BL. Later it disappeared and presumably was removed by regenerating cells. Alveolar macrophages were commonly seen in alveolar spaces on the third day following injury.

One week following injury, the foci of damage and ongoing regeneration were more difficult to locate on random tissues blocks. When seen, they showed changes similar to those observed at 72 hours. Cells as described before were apparently repopulating the capillary BL tube and the surfaces of epithelial BL. In addition, another cell type became prominent: in the interstitium, which following injury contained principally collagen and elastic fibers, cells began to appear containing many free ribosomes and multiple dense bodies, apparently lysozomes. Although these cells touched in spots the connective tissue surface of epithelial BL they generally did not show the tendency to align themselves along the BL. These cells probably represent regenerating septal cells (Fig. 3). Intra-alveolar macrophages again were prominent.

At *two weeks* most sections revealed relatively normal lung. The rare abnormal sites contained intact but pleated epithelial BL, disrupted endothelial BL and cells which aligned along the denuded surfaces of epithelial BL. Septal cells were present and resembled those described earlier. A new finding was the presence of collagen and long, slender cells within alveolar spaces. This became more prominent in the specimen removed at three weeks (Fig. 4) although the frequency of such foci was markedly decreased. In such sites the epithelial BL was still intact and provided a scaffolding for proliferating epithelial cells. The capillary BL had largely disappeared except for small aggregates of BL like material. Cells which by location and appearance would suggest regenerating endothelial cells were uncommon. Elastic fibers were seen only in the interstitium associated with bundles of collagen.

Discussion

To preserve the three-dimensional pattern of a tissue after cell death, and to serve as a scaffolding for regenerating cells appear to be common and important properties of BL.

It was shown by Volkmann (1893) that injured skeletal muscle will completely regenerate and become functional only if the sarcolemma remained intact; if it was damaged, the injury healed with a scar. Later Clark (1946) in an ingenious experiment demonstrated that regeneration of skeletal muscle fibers occurred in the direction of old fibers even if a piece of muscle was excised and reimplanted at right angles to its original orientation. When with the advent of electron microscopy the principal component of sarcolemma was identified as BL, it became apparent that BL is the guide for orderly positioning of regenerating muscle cells and that its presence is necessary for reestablishment of the original structures. Very similar events occur in Wallerian regeneration of nerves (O'Daly and Imaeda, 1967) as well as during repair of tubular necrosis. In crush injury of cutaneous nerves the Schwann cells and axons regenerate inside the BL envelope which has been left behind by the previously damaged cells. Following temporary occlusion of the renal pedicle (Cuppage *et al.*, 1967) and following injury with mercuric chloride (Cuppage *et al.*, 1969) the tubular BL remains intact after cell death as a structural framework for regenerating epithelial cells. Disruption of BL induces disorganized proliferation of epithelium and apparently requires interaction of epithelial and mesenchymal elements to patch up the defect (Oliver, 1953). In a similar sequence of events the repopulation of skin autografts and homografts begins as epithelial outgrowth from the hair follicles of the

graft. The new cells advance over the BL left in situ by the dislodged epidermis (Giacometti and Parakkal, 1969; Krowczyk, 1971).

Events similar to those described for regeneration of muscle, kidneys, nerves and epidermis take place in the lung following injury with oleic acid. Five hours after intravenous injection of oleic acid the epithelial, endothelial and interstitial cells in large portion of the lung parenchyma disentigrate into cell debris. The epithelial and capillary BL remain intact (Fig. 1–4). They retain, as a microskeleton, the framework of alveolar structure and provide undisrupted structural continuity between the living and dead portions of the lung. They also serve as a scaffolding for regenerating epithelial and endothelial cells. These cells begin to repopulate the epithelial and capillary BL soon after injury and with their pseudopods unfold the BL pleats in areas where it has collapsed (Fig. 2, 3, 4). These developments lead within three weeks to reestablishment of relatively normal lung parenchyma.

It is unlikely that the nature of injury used in this experiment possesses the unique property of preserving BL. While the exact nature of toxic effects of oleic acid are unknown, it has been shown to be a highly necrotizing agent (Sevitt, 1962). When injected intravascularly or subcutaneously it will cause marked necrosis wherever it lodges and it has been suspected of contributing to injury of pulmonary fat embolism (Rubia and Schulz, 1963; Sevitt, 1962). Oleic acid could not be visualized on electromicrographs nor was excessive lipid apparent with the light microscope. The fate of oleic acid is therefore unknown and the exact nature of injury remains unexplained.

Of considerable interest is the cellular response to injury. It was characterized during the first 72 hours by focal infiltration with polymorphonuclear leucocytes and with beginning proliferation of epithelial and endothelial cells. These grew along the alveolar surface of epithelial and endothelial BL and became the predominant cell types in the one and two weeks old lesions. By that time the polymorphonuclear leucocytes had almost disappeared. The parenchymal cells were frequently seen in apposition with cell debris which they apparently removed. The mechanism of the removal was not clear since phagocytic activity was not prominent nor did these cells contain a notable complement of lysozomes. The epithelial cells and endothelial cells aligned themselves with their respective BL, sent out cell processes into the BL pleats wherever it has folded and removed cell debris which became trapped between BL and cell bodies. In no instance have these cells penetrated the BL or proliferated on the connective tissue surface of BL.

In the later stages of regeneration, two and three weeks following injury, only rare foci of damage remained. They now contained collagen and fibroblast like cells in the alveolar spaces in addition to alveolar macrophages and regenerating epithelial cells which continued to cover the denuded BL. Their presence as well as the preservation of epithelial BL strongly indicated that regeneration was continuing inspite of beginning intraalveolar fibrosis.

The regenerating epithelial and endothelial cells of the lung did not produce a new layer of BL but used the old, denuded BL for their immediate footing and support. The overall investment of BL thickness remained therefore unchanged after completion of regeneration. This differs substantially from the repair of other organs. Epithelial cells of the nephron (Cuppage *et al.*, 1967) deposit a new layer of BL as do cutaneous nerves (O'Daly and Imaeda, 1967), skeletal muscle

cells (Price *et al.*, 1964) and skeletal muscle capillaries (Vracko and Benditt, 1970). Following regeneration these structures have two or more layers of BL, depending upon the number of cell generations, and their BL has a thickened, split or lamellated appearance.

The present study suggests that intact BL scaffolding is required for orderly repair of the lung since cell replacement occurred only along preformed BL. If this observation is correct then disruption of epithelial BL could prevent reestablishment of epithelial continuity: Interalveolar gaps would result, enlarging individual air space giving the lung the characteristic appearance of emphysema. Since emphysema became apparent in the lungs of dogs sacrificed at two and three weeks following injury it is conceivable that it was caused by disruptions of BL which occurred as the result of injury with oleic acid. Such disruptions of BL could however, not be identified with the electron microscope.

It was noted that the durability of denuded epithelial BL exceeded that of capillary BL. The latter became discontinuous two weeks after injury in segments which were not joined with the epithelial BL. Within the few unhealed foci three weeks after injury the capillary BL was generally absent from interstitial spaces as a well defined continuous sheet (Fig. 4). It remained only in form of small discontinuous aggregates of BL material which perhaps represented cross-sections of an irregular, net-like structure with the overall configuration of a capillary. In lesions in which the endothelial BL was disappearing, the epithelial BL remained intact without morphological evidence of damage (Fig. 4).

The relationship between denuded BL, which has survived injury and regenerating cells appears to be governed by at least two important biologic principles, both of which are not well understood. One of these has to do with polarity of BL while the other is concerned with BL specificity for cell types. It is apparent from this and other studies (Vracko and Benditt, 1970) that parenchymal cells which regenerate in damaged lung and skeletal muscle grow exclusively on surfaces of BL from which previous cell generation have shed. It is conceivable that in the lung model the epithelial and endothelial cells do not have access to the opposite BL surface. In previous experiments however, which were concerned with regeneration of excised and reimplanted pieces of skeletal muscle (Vracko and Benditt, 1970) the regenerating skeletal muscle cells and capillaries did have access to the connective tissues side of BL yet regeneration occurred invariably within the confines of BL tubes, indicating that cells "recognize" the cell surface of BL.

The second recognition system which appears to exist between denuded BL and regenerating cells is concerned with specificity of BL for cell types. In regenerating lung and skeletal muscle (Vracko and Benditt, 1970) the cells which grew along a BL were always the cell type which was supported by that particular BL before injury. While it is conceivable that BL possesses the capacity to induce "uncommitted" cells to mature into cell types which are appropriate for that particular BL it is more likely, that cell specific markers exist on the surface of BL which are recognized by cells and which identify the BL as belonging to a particular cell type. The specific cells then proceed to multiply until they have covered the available surface of "their" BL.

If this general concept is correct then BL should be viewed as a microskeleton which provides the scaffolding for regenerating cells. If cells drop out by the process of normal cell turn-over or following certain types of injury the replacement by new cell generations occurs along the denuded surfaces of BL. In this manner complete regeneration of complex organs, composed of different types of parenchymal cells can occur provided the integrity of BL is maintained.

Regardless of the exact nature of the forces acting upon the relationships between BL and regenerating cells, BL of lung as well as other tissues seems to be responsible for orderly regeneration of organs and tissues in harmonious continuity with adjacent, undamaged structures and for the re-establishment of function.

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References

Clark, W. E.: An experimental study of the regeneration of mammlian striped muscle. J. Anat. (Lond.) **80**, 24 (1946).

Cottrell, T. S., Levine, O. R., Senior, R. M., Wiener, J., Spiro, D., Fishman, A. P.: Electron microscopic alterations at the alveolar level in pulmonary edema. Circulat. Res. **21**, 783 (1967).

Cuppage, F. E., Cunningham, N., Tate, A.: Nucleic acid synthesis in the regenerating nephron following injury with mercuric chloride. Lab. Invest. **21**, 449 (1969).

Cuppage, F. E., Neagoy, D. R., Tate, A.: Repair of nephron following temporary occlusion of the renal pedical. Lab. Invest. **17**, 660 (1967).

Giacometti, L., Parakkal, P. L.: Skin transplantation: orientation of epithelial cells by the basement membrane. Nature (Lond.) **223**, 514 (1969).

Krawczyk, W. S.: A pattern of epidermal cell migration during wound healing. J. Cell Biol. **49**, 247 (1971).

O'Daly, J. A., Imaeda, T.: Electron microscopic study of Wallerian degeneration in cutaneous nerves caused by mechanical injury. Lab. Invest. **17**, 744 (1967).

Oliver, J.: Correlations of structure and functions and mechanisms of recovery in acute tubular necrosis. Amer. J. Med. **15**, 535 (1953).

Price, H. M., Howes, L. E., Blumberg, J. M.: Ultrastructural alterations in skeletal muscle fibers injured by cold. Lab. Invest. **13**, 1264 (1964).

Rubia, F. J., Schulz, H.: Elektronenmikroskopische Untersuchungen des Blut-Luft-Weges bei der experimentellen Fettembolie der Lunge. Beitr. path. Anat. **128**, 78 (1963).

Sevitt, S.: Fat embolism, p. 63. London: Buttersmiths 1962.

Volkmann, R.: Über die Regeneration des quergestreiften Muskelgewebes beim Menschen und Saugethier. Beitr. path. Anat. **12**, 23 (1893).

Vracko, R.: Skeletal muscle capillaries in diabetes. Circulation **41**, 271 (1970).

Vracko, R., Benditt, E. P.: Capillary basal lamina thickening: its relationship to endothelial cell death and replacement. J. Cell Biol. **47**, 281 (1970).

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